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Effect of pH on the Efficiency of Growth by Pure Cultures of Rumen Bacteria in Continuous Culture

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A total of 10 strains of rumen bacteria, *Selenomonas ruminantium* HD4, *Megasphaera elsdenii* B159, *Butyrivibrio fibrisolvens* A38, *Streptococcus bovis* JB1, *Lactobacillus vitulinus* GA1, *Bacteroides ruminicola* B_14, *B. ruminicola* GA33, *Ruminococcus albus* 7, *Ruminococcus flavefaciens* C94, and *Bacteroides succinogenes* S85, were grown in energy-limited continuous cultures at a dilution rate of approximately 0.165 h^{-1}. Periodically, the pH of the medium reservoir was lowered approximately 0.3 pH units, and the energy source concentration remaining in the culture vessel, optical density, cell mass, and pH were determined. A low pH appeared to have a detrimental effect on cell yields. Large variations were seen among strains in both the magnitude of yield depressions at lower pH values and in the pH at which the culture washed out. Lactate analysis indicated that lactate production in *S. bovis* was increased at pH values below 5.75. The data are discussed in relation to the effect of pH on the efficiency of protein synthesis in the rumen and rumen microbial ecology.

The rumen, although well buffered by bicarbonate, phosphate, protein, and volatile fatty acids, can vary in pH from approximately 7.0 to less than 5.0 under different dietary conditions. In vivo (2, 11, 14, 22) and in vitro (8, 9) observations have indicated that the relative success of rumen bacteria is correlated with pH, and recent work has shown that an acidic environment can decrease the maximum growth rate of rumen bacteria (20).

The detrimental effects of low pH on bacterial growth are well documented (3), but the mechanisms involved are not well understood. According to the chemiosmotic theory (15), the inhibition of growth at a low pH could arise from insufficient energy to shift protons outwardly through the cell membrane to establish a proton motive gradient (7). As it has also been accepted by many that membranes may not be impermeable to protons (25), the partial inhibition of growth could result from an additional expenditure of energy to maintain the membrane potential.

Generally, a near-neutral intracellular pH is maintained in bacteria (7), but the intracellular pH can decrease considerably when the cell is subjected to an acidic environment (16). As many enzymes are markedly sensitive to pH, the growth inhibitions that are seen at a low pH could be caused by a direct effect of the H ion on cellular components. Such direct effects would not necessarily cause a decrease in the efficiency of growth.

As the ruminant animal is largely dependent on microbes as a protein source, the efficiency of rumen microbial growth is of critical importance to ruminant performance. The experiments described herein were performed to examine the effect of pH on the efficiency of energy utilization for growth by rumen bacteria. It was also hoped that these experiments might provide information about the mechanism of growth inhibition by adverse pH in these bacteria.

MATERIALS AND METHODS

Organisms. *Selenomonas ruminantium* HD4, *Megasphaera elsdenii* B159, *Butyrivibrio fibrisolvens* A38, *Streptococcus bovis* JB1 (17), *Lactobacillus vitulinus* GA1, *Bacteroides ruminicola* GA33 and B_14, *Ruminococcus albus* 7, *Ruminococcus flavefaciens* C94, and *Bacteroides succinogenes* S85 were used.

Media and cell growth. The media and conditions of cell growth were essentially the same as those previously described (18), except the concentration of ammonia was doubled, a single dilution rate was used, and the pH of the medium reservoir was lowered by approximately 0.3 pH units every 24 h with concentrated HCl (except for the incubation shown in Fig. 11). For the incubations shown in Fig. 1 to 10, the dilution rate was set at approximately 0.165 h^{-1} with the flowmeter of the chemostat. This dilution rate was used because it provided a rather low growth rate that allowed a 98% exponential turnover each 24 h. Previous work has indicated that a steady-state optical density is achieved by such a turnover if the change in culture conditions is not drastic (18). The concentration of the limiting energy source, glucose or cellobiose, is indicated in each figure legend. The washout point was defined as the pH at which the optical density of the culture vessel was equal to that of the media in the reservoir.

Sampling and substrate analyses. After 24 h at

a given pH, a sample was withdrawn from the culture vessel, was immediately passed through a 0.65-μm-pore size membrane filter (Millipore Corp., Bedford, Mass.), and was stored at −15°C until analyzed for the concentration of carbohydrate. Another 50-ml sample (25 ml each for *S. bovis* and *B. ruminicola*) was removed from the culture vessel, and the bacterial dry weight was determined by the membrane filter method of Isaccson et al. (12). Cell yields were defined as the gram dry weight of cells divided by the difference in carbon source concentrations between the culture and reservoir vessels. The pH and optical density (Gilford spectrophotometer model 250 at 600 nm) of the culture were also measured with subsequent samples. When sampling was completed, concentrated HCl was added to the medium reservoir to lower the pH.

The affinity of *R. flavefaciens* for cellobiose (see Fig. 11) was determined by methods that have previously been outlined (18). The substrate sample was later analyzed for glucose, cellobiose, or lactate by methods that have previously been described (19). The standard error of the mean of each dilution rate (see Fig. 1 to 10) or a linear regression of the Lineweaver-Burk plot (see Fig. 11) is given in each figure legend (23).

RESULTS

The continuous culture of *S. ruminantium* is shown in Fig. 1. The dry weight of cells remained constant until the pH was lowered to 6.0 (Fig. 1). As the utilization of glucose was nearly complete at each pH, the yield of cells decreased below pH 6.0. At pH 4.85 the growth rate could no longer equal the dilution rate, and washout occurred. Because *S. ruminantium* has been shown to switch its fermentation products from lactate to acetate-propionate as a function of growth rate (21), lactate was measured in the substrate samples. The lactate concentrations (Table 1) remained at a low level and did not seem to vary as a function of pH, but at pH 5.0, lactate levels, although still low, were approximately double the values that had been seen at higher pH's.

S. bovis (Fig. 2) and *L. vitulinus* (Fig. 3) were able to tolerate a wider range of pH's before washout, but in each case a low pH was associated with lower cell yields. Because *S. bovis* has been shown to switch its fermentation products from lactate to acetate-ethanol and to obtain higher growth yields (19), lactate was assayed from the substrate sample. In this case (Table 1), the lower pH (less than 5.75) values were

TABLE 1. *Ratio of lactate production (g/liter) to glucose utilization (g/liter) by S. ruminantium and S. bovis at various pH's*

pH (±0.05)	*S. ruminantium*	*S. bovis*
6.75	0.095	0.039
6.65	0.062	
6.50		0.035
6.35	0.064	
6.15	0.067	0.037
5.75	0.074	0.035
5.40	0.074	
5.25	0.082	0.107
5.10	0.078	
5.00	0.192	0.274
4.80		0.463
4.75		0.692

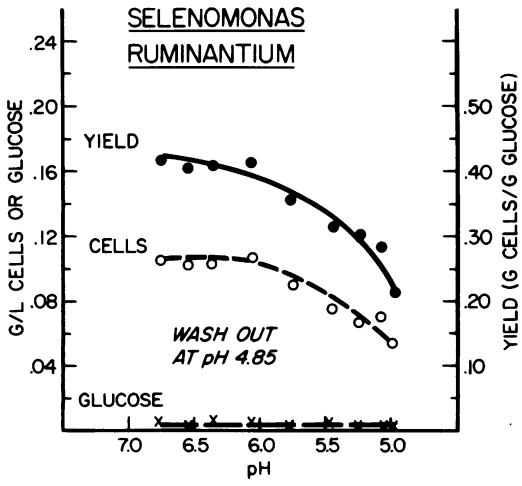

FIG. 1. *Growth of S. ruminantium at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.251 g/liter, and the dilution rate was maintained at 0.155 ± 0.003 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

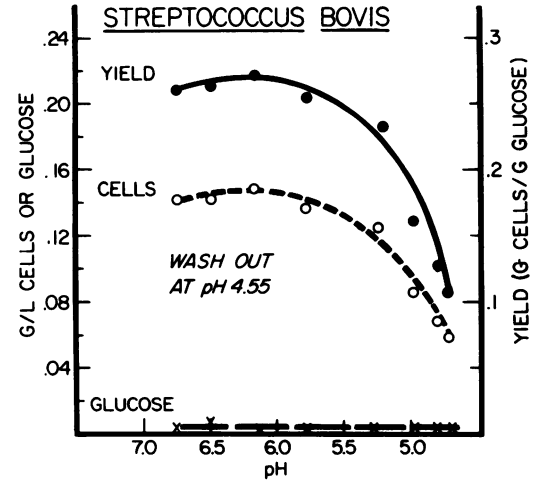

FIG. 2. *Growth of S. bovis at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.540 g/liter and the dilution rate was maintained at 0.158 ± 0.004 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

associated with significant increases in lactate.

The relationship between cell yield and pH was more complex for *M. elsdenii* (Fig. 4). The dry weight of the cells increased, glucose utilization remained rather constant, and the cell yield increased between pH 6.70 and 5.75. When the pH was decreased further, however, the cell mass declined, and glucose accumulated in the culture. The rate of decrease in cell mass was greater than the increase in residual glucose, and the cell yield declined at values lower than pH 5.5. Washout occurred at pH 4.90.

B. ruminicola B_14 showed a slight increase in cell yield when the pH was lowered from 6.75 to 6.50, but the yield declined at pH 5.50 and 5.25 (Fig. 5). Glucose accumulated at pH 5.25, and washout occurred at pH 5.10. Comparison of the B_14 strain (Fig. 5) with the GA33 strain (Fig. 6) indicated that strain differences can occur. GA33

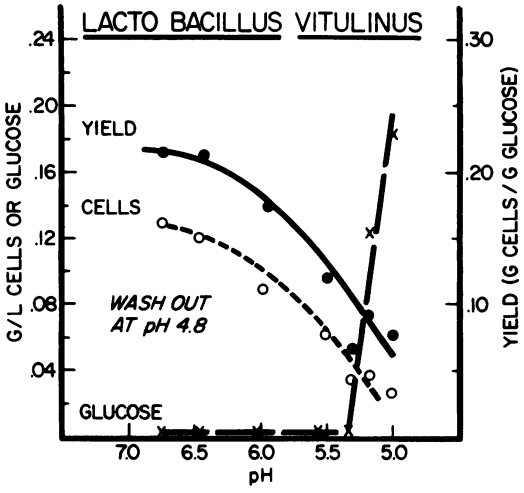

FIG. 3. *Growth of L. vitulinus at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.521 g/liter, and dilution rate was maintained at 0.169 ± 0.005 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

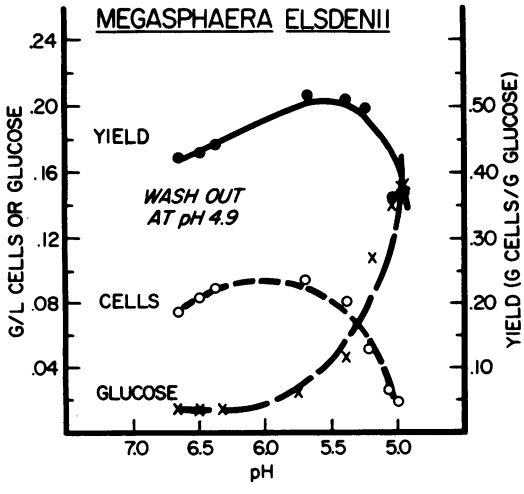

FIG. 4. *Growth of M. elsdenii at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.199 g/liter, and the dilution rate was maintained at 0.173 ± 0.001 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

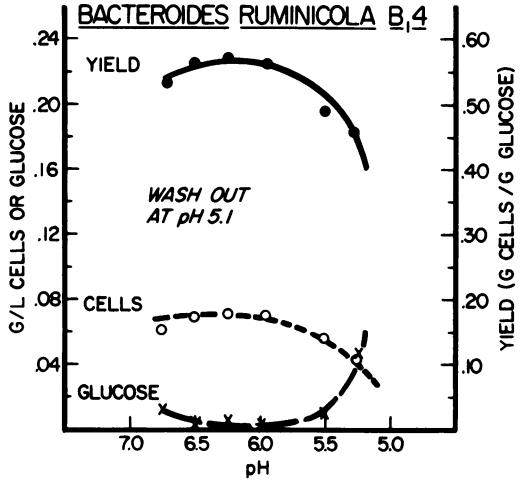

FIG. 5. *Growth of B. ruminicola B_14 at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.242 g/liter, and the dilution rate was maintained at 0.168 ± 0.006 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

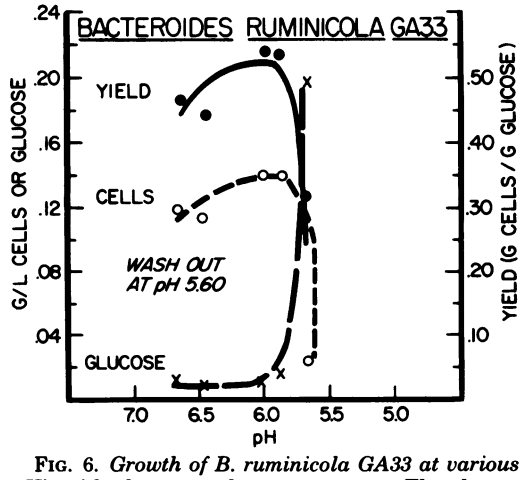

FIG. 6. *Growth of B. ruminicola GA33 at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.264 g/liter, and the dilution rate was maintained at 0.169 ± 0.002 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

appeared to be more susceptible to a low pH and washed out at pH 5.60.

Cellulolytic bacteria (Fig. 7 to 10) washed out at high pH values. *B. fibrisolvens* (Fig. 7) was the most resistant cellulolytic species, but even this culture washed out at pH 5.70. *B. fibrisolvens* (Fig. 7), *R. albus* (Fig. 9), and *R. flavefaciens* (Fig. 10) showed lower cell yields at pH values close to washout, but the decline in yield as a function of pH was abrupt. *B. succinogenes* (Fig. 8) showed depressed cell dry weights at pH values close to washout, but the cell yield did

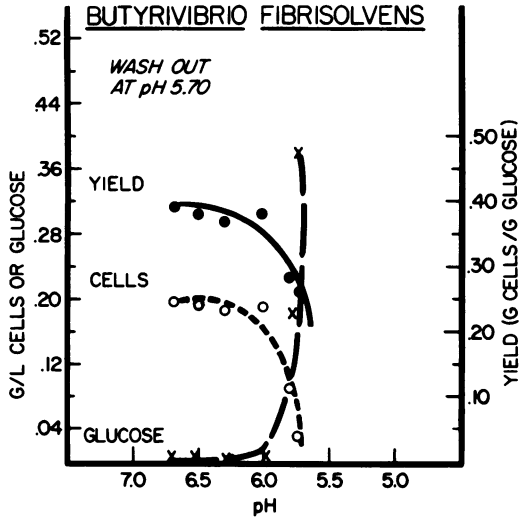

FIG. 7. *Growth of B. fibrisolvens at various pH's with glucose as the energy source. The glucose concentration of the medium reservoir was 0.496 g/liter, and the dilution rate was maintained at 0.162 ± 0.004 h^{-1}. The steady state level of cells (○) and glucose (×) within the culture vessel and yield (●) are shown.*

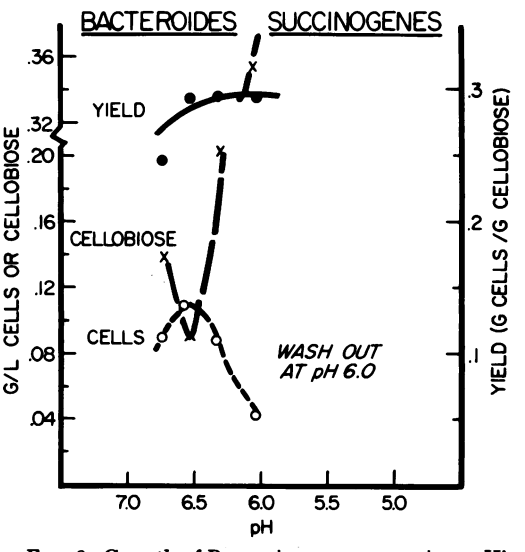

FIG. 8. *Growth of B. succinogenes at various pH's with cellobiose as the energy source. The cellobiose concentration of the medium reservoir was 0.494 g/liter, and the dilution rate was maintained at 0.169 ± 0.004 h^{-1}. The steady state level of cells (○) and cellobiose (×) within the culture vessel and yield (●) are shown.*

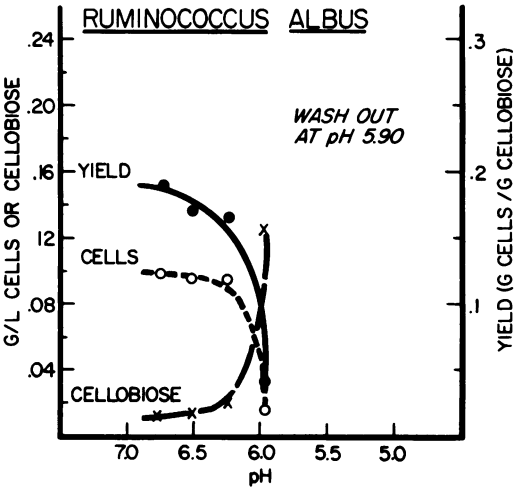

FIG. 9. *Growth of R. albus at various pH's with cellobiose as the energy source. The cellobiose concentration of the medium reservoir was 0.533 g/liter, and the dilution rate was maintained at 0.171 ± 0.008 h^{-1}. The steady state level of cells (○) and cellobiose (×) within the culture vessel and yield (●) are shown.*

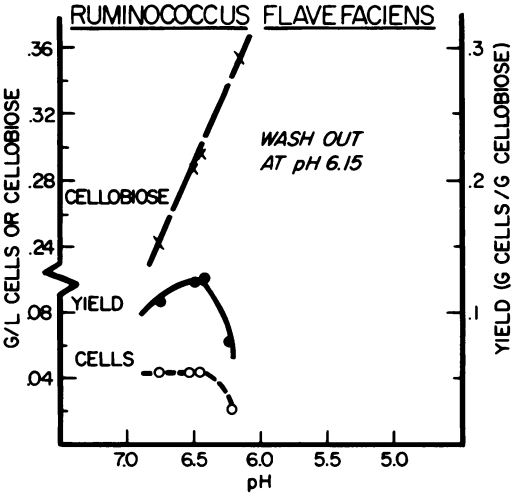

FIG. 10. *Growth of R. flavefaciens at various pH's with cellobiose as the energy source. The cellobiose concentration of the medium reservoir was 0.614 g/liter, and the dilution rate was maintained at 0.170 ± 0.008 h^{-1}. The steady state level of cells (○) and cellobiose (×) within the culture vessel and yield (●) are shown.*

not decline. Both *B. succinogenes* and *R. flavefaciens* (Fig. 8 and 10) left relatively large amounts of cellobiose in the culture medium even when the pH was high. A Lineweaver-Burk plot of 1/substrate concentration versus 1/growth rate for *R. flavefaciens* (Fig. 11) indicated that this strain had a low affinity for cellobiose with a K_s of 0.238 g/liter or 0.67 mM.

DISCUSSION

The dilution rates that were used in the incubations shown in Fig. 1 to 10 were all substantially lower than the maximum growth rates of each strain. However, it should be recognized that these dilution rates are higher than solid dilution rates that have been reported for the rumen (10). Therefore, strains may exist in the rumen at lower pH's than when those washouts occurred. Lower dilution rates were not used because it was felt that cell lysis might confound the interpretation of the cell yield data.

Lower pH environments can substantially decrease the cell yields of pure cultures of rumen bacteria. Even *S. bovis* and *L. vitulinus*, which tolerate a lower pH than other species, showed substantially depressed cell yields at a low pH. Stewart (24) reported that numbers of filter paper-degrading bacteria during in vitro rumen fluid incubations were depressed when the pH declined. These results are consistent with the high pH washout values that were seen with the cellulolytic bacteria (Fig. 8 to 10). *B. fibrisolvens* was the cellulolytic species most resistant to a low pH, and this feature is probably advantageous to its niche as a soluble carbohydrate utilizer as well.

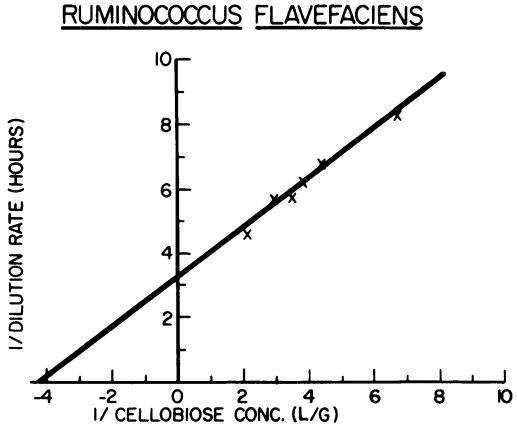

FIG. 11. *Lineweaver-Burk plot of the growth of R. flavefaciens on cellobiose. The cellobiose concentration of the medium reservoir was 0.673 g/liter, and the pH always remained between 6.75 and 6.50.*

As many of these strains have fastidious nutritional requirements (4), it was necessary to use a complex medium, which made a detailed analysis of fermentation products difficult. Lactate measurement was possible, and as switches from lactate to other fermentation products have been reported for *S. ruminantium* (19) and *S. bovis* (20), this analysis was performed. *S. ruminantium* produced only small amounts of lactate regardless of pH (Table 1). Lactate production by *S. bovis*, however, did show pH dependency (Table 1), and lactate accumulated at pH values below 5.2. Lactate production by *S. bovis* appears to be a complex function of both growth rate (19) and pH. As *S. bovis* has been implicated in the onset of rumen acidosis (11), this interaction may warrant further study.

The high level of cellobiose left in the culture vessel by *R. flavefaciens* at pH 6.75 (Fig. 10) was surprising. The accumulation of the energy source at a low dilution rate indicates that some component of the medium other than the energy source is limiting or that the culture has a low affinity for the energy source. For ascertaining which factor was involved, cellobiose levels in the culture vessel were measured at a variety of dilution rates. A Lineweaver-Burk plot of these residual cellobiose concentrations (Fig. 11) indicated that the affinity was limiting growth. The C94 strain of *R. flavefaciens* does not ferment glucose or many other substrates (5) and as its cellobiose affinity is low relative to other rumen bacteria (18), mechanisms must be present to protect the cellobiose yielded by cellulose degradation from other bacteria. The close physical attachment of cellulolytic bacteria to cellulose fibers (1, 13) may provide such a mechanism of protection.

The accumulation of the energy source in the culture vessel as a function of pH also occurred in some incubations (Fig. 3 to 10). According to the Michaelis-Menten kinetics equation $v = [V_{max}S/(K_m + S)]$, such accumulations could either result from a decrease in the maximum velocity (maximum growth rate) or from a decrease in the affinity because the velocity (growth rate) was held constant in these experiments. The maximum velocity of some of these strains has been shown to vary according to pH (20), but the possibility exists that the pH could also be affecting substrate affinity.

As detailed fermentation balances could not be calculated from these experiments, it is impossible to say that changes in fermentation products were not at least partially responsible for the depressed growth yields that were seen at low pH's. Indeed, lactate accumulation in the low pH incubations of *S. bovis* probably did contribute to decreasing the yield, but the mag-

nitude of the depression is too great to be explained by the difference in the adenosine 5'-triphosphate yield between an acetate-ethanol fermentation and a lactate fermentation (19). Such changes in fermentation were not responsible for the reduced growth yields of *S. ruminantium*, and as declines in yield were often great for the other strains, it seems likely that a low pH is detrimental to the efficiency of energy utilization for growth in the majority of these rumen bacteria.

If the inability of an organism to grow at a low pH were solely mediated by the negative effect of acid on an enzyme or transport protein, one would not expect the efficiency of growth to be significantly affected at a constant growth rate. The cellulolytic bacteria (*B. fibrisolvens*, *B. succinogenes*, *R. albus*, *R. flavefaciens*), *B. ruminicola* GA33, and *M. elsdenii* showed abrupt sensitivity to decreasing pH, and often a decline of 0.25 units made the difference between maximum cell yield and washout of the culture. *B. succinogenes* did not show depressed yields before washout. These abrupt transitions to washout would be consistent with a direct effect on a cellular constituent. In the cases of *S. ruminantium*, *S. bovis*, and *L. vitulinus*, very gradual declines in cell yield were seen as a function of decreasing pH. Such gradual declines in cell yield would be consistent with an uncoupling of growth by a chemiosmotic mechanism. Uncoupled growth has been shown to occur in other organisms (25).

In a previous experiment (20), *M. elsdenii*, *S. ruminantium*, and *S. bovis* did not grow in batch culture at as low a pH as they did in these continuous cultures. These batch culture incubations were performed with unadapted inocula grown at a pH of approximately 6.75. These differences (as much as 0.4 pH units) may indicate that an adaptation is required for an organism to grow at an adverse pH.

Buffers have been used in ruminant diets to correct acidotic symptoms due to lactate and to increase milk fat production (25), but little attention has been given to the effect of rumen buffers on the efficiency of microbial protein synthesis. As the bacteria that were able to maintain themselves in the chemostat cultures at low pH's (*S. bovis*, *L. vitulinus*, *S. ruminantium*, *M. elsdenii*, and *B. ruminicola* B_14) all showed significantly depressed yields at these pH's, it seems likely that pH is an effector of overall microbial protein synthesis in the rumen. Since the rumen pH of animals fed high starch diets is often below 6.0, it is possible that the availability of microbial protein to the animal could be enhanced by a manipulation of the rumen with dietary buffers.

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